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Naturally Occurring Human Genetic Variation in the 3′-Untranslated Region of the Secretory Protein Chromogranin A Is Associated With Autonomic Blood Pressure Regulation and Hypertension in a Sex-Dependent Fashion

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Objectives We aimed to determine whether the common variation at the chromogranin A (*CHGA*) locus increases susceptibility to hypertension.

Background *CHGA* regulates catecholamine storage and release. Previously we systematically identified genetic variants across *CHGA*.

Methods We carried out dense genotyping across the *CHGA* locus in >1,000 individuals with the most extreme blood pressures (BPs) in the population, as well as twin pairs with autonomic phenotypes. We also characterized the function of a trait-associated 3′-untranslated region (3′-UTR) variant with transfected *CHGA* 3′-UTR/luciferase reporter plasmids.

Results *CHGA* was overexpressed in patients with hypertension, especially hypertensive men, and *CHGA* predicted catecholamines. In individuals with extreme BPs, *CHGA* genetic variants predicted BP, especially in men, with a peak association occurring in the 3′-UTR at C+87T, accounting for up to ~12/~9 mm Hg. The C+87T genotype predicted *CHGA* secretion in vivo, with the +87T allele (associated with lower BP) also diminishing plasma *CHGA* by ~10%. The C+87T 3′-UTR variant also predicted the BP response to environmental (cold) stress; the same allele (+87T) that diminished basal BP in the population also decreased the systolic BP response to stress by ~12 mm Hg, and the response was smaller in women (by ~6 mm Hg). In a chromaffin cell-transfected *CHGA* 3′-UTR/luciferase reporter plasmid, the +87T allele associated with lower BP also decreased reporter expression by ~30%. In cultured chromaffin cells, reducing endogenous *CHGA* expression by small interfering ribonucleic acid caused approximately two-thirds depletion of catecholamine storage vesicles.

Conclusions Common variant C+87T in the *CHGA* 3′-UTR is a functional polymorphism causally associated with hypertension especially in men of the population, and we propose steps ("intermediate phenotypes") whereby in a sex-dependent fashion this genetic variant influences the ultimate disease trait. These observations suggest new molecular strategies to probe the pathophysiology, risk, and rational treatment of hypertension. (J Am Coll Cardiol 2008;52:1468–81) © 2008 by the American College of Cardiology Foundation

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Chromogranin A (*CHGA*), a 48-kDa acidic polypeptide (1,2), is the major protein costored and coreleased with catecholamines from secretory vesicles in adrenal medulla and postganglionic sympathetic axons (3). Catecholamine storage vesicles (or chromaffin granules) of the adrenal medulla contain remarkably high concentrations of *CHGA*, catecholamines, adenosine triphosphate, and Ca^{2+}, and *CHGA* seems to bind and store both catecholamines and Ca^{2+} (4). *CHGA* also binds to the vesicle membrane, where it may influence the release of calcium from secretory granules to the cytosolic exocytotic machinery through the inositol 1,4,5-trisphosphate receptor/Ca^{2+} channel (5). *CHGA* is required for formation of catecholamine secretory vesicles in chromaffin cells, and its expression may be sufficient to induce a regulated secretory system in nonsecretory cells (6). *CHGA* is also a pro-hormone that gives rise to biologically active peptides such as the dysglycemic peptide pancreastatin (7,8), the antimicrobial peptide chromacin (9), the vasodilator vasostatin (10), and catestatin, which acts to inhibit catecholamine release (11,12).

Essential hypertension is a complex trait (13), with contributions from multiple factors: cardiovascular, neuronal, renal, and adrenal. Over the past ~20 years, phenotypic links between *CHGA* and essential (idiopathic, genetic) human (14–17) and rodent (18) hypertension have been repeatedly observed. Plasma *CHGA* concentration correlates with catecholamine release rates (19), and increases in blood pressure (BP) caused by the action of catecholamines are likely to be coupled to the formation of dense-core secretory granules, whose biogenesis is regulated in vivo by *CHGA* (20). Recently, we systematically identified common genetic variation in human *CHGA* by resequencing the gene in several human populations (21); here we explored whether common genetic variation at the *CHGA* locus is associated with BP, beginning with a large population-based sample of extreme BPs, in which we found that a 3′-untranslated region (3′-UTR) polymorphism (C+87T) is substantially associated with both diastolic blood pressure (DBP) and systolic blood pressure (SBP). We then established its influence on an earlier pathogenic phenotype (environmental stress-evoked change in BP), and finally documented its effect on gene expression in a transfected reporter system.

Abbreviations and Acronyms

BP = blood pressure
CHGA = chromogranin A
DBP = diastolic blood pressure
LD = linkage disequilibrium
PCR = polymerase chain reaction
SBP = systolic blood pressure
siRNA = small interfering ribonucleic acid
SNP = single nucleotide polymorphism
SNPEM = single nucleotide polymorphism expectation maximation
3′-UTR = 3′-untranslated region

Methods

Subjects and Clinical Characterization

Hypertension. DIAGNOSIS OF HYPERTENSION. Since hypertension is part of a larger syndrome, all individuals of diverse ancestries were phenotyped for not only BP, but also associated traits, both metabolic and renal (Online Tables 1 and 2).

PHENOTYPE (*CHGA*, CATECHOLAMINE) AND BP STUDY. In the first (purely phenotypic) study, we measured the plasma concentrations of *CHGA*, norepinephrine, lipids, and creatinine (see the following text) in 724 individuals with normal renal function (serum creatinine ≤1.5 mg/dl), stratified by BP status: normal BP (documented at <135/<85 mm Hg, on no medications), versus a diagnosis of essential hypertension (documented at DBP ≥90 mm Hg). Of those with hypertension, 75% were treated with antihypertensive medications. BPs were determined in triplicate (and then averaged) in seated subjects with an oscillometric device (DynaPulse, PulseMetric, Vista, California), validated, and calibrated as described previously (22). During the same visit, the same subjects also underwent prolonged (5-min, ~400 beats), noninvasive monitoring of BP with a radial artery applanation tonometer (Colin Pilot, Colin Instruments, San Antonio, Texas); such prolonged radial arterial readings correlated with both SBP (Spearman rho = 0.57; $p < 0.001$) and DBP (Spearman rho = 0.53; $p < 0.001$) obtained by the DynaPulse brachial cuff.

CHGA GENOTYPE AND BP STUDY. In the second (genotype/phenotype) study, a population cohort with extreme BPs consisted of 470 male and 558 female white (European ancestry, by self-identification) subjects. These participants were selected based on DBP in the upper or lower most extreme (fifth) percentiles of DBP distribution in 25,599 men and 27,479 women in a primary care practice at Kaiser-Permanente of Southern California medical group (23,24). Subjects were ascertained on the DBP trait, because twin and family studies have provided evidence that DBP is substantially heritable (25), and SBP correlates highly with DBP. BP was measured in seated individuals with an aneroid sphygmomanometer in a single health appraisal clinic site by trained, long-term personnel, and BP measurement was repeated if elevated on initial reading. The health appraisal visit included measurement of vital signs, extended questionnaire, and clinical laboratory evaluation, including hemogram, chemistry panel, glucose, and lipids. Individuals in the upper DBP percentiles were age-matched to subjects in the lower extreme percentiles. We ascertained 189 men (age 58.5 ± 10.4 [SD] years) with DBP ≥96 mm Hg and 281 men (age 57.7 ± 15.8 years) with DBP ≤61 mm Hg. Among the women, 175 were ascertained with high DBP (≥92 mm Hg; age 61.4 ± 11.2 years), along with 383 age-matched women with low DBP (DBP ≤59 mm Hg; age 53.9 ± 14.0 years). BP was treated by antihypertensive medications in 48% of subjects with hypertension. Thus, the DBP group separation for men was >35 mm Hg, while that for women was >33 mm Hg. Power calculations were performed using the on-line genetic power calculator for quantitative trait loci (26) according to the method described by Schork et al. (27). The power of an association

study on the extreme samples was computed under varying disease allele frequencies for type I error rates of 0.05, 0.001, or 0.00000001 ("genome-wide" level), for recessive, additive, and dominant models of inheritance, using the proportion of variance of BP explained by the locus at 0.5%, 1%, 2.5%, and 5%, and assuming the marker locus is the actual trait locus ($D' = 1.0$). At a genome-wide alpha of $p < 10^{-8}$, we determined that this sample has >90% power to detect genotype association with BP, if the genotype at minor allele frequency ≥15% contributes ≥2.5% of total variance.

Twin pairs. Twin subject characteristics are described in our previous reports (28). Twin pairs, age 15 to 84 years (median 40 years), were 69% monozygotic and 31% dizygotic. Twin zygosity was confirmed by single nucleotide and microsatellite polymorphisms, as previously described (25,29,30); 9.9% of the twins were hypertensive (8.8% treated with antihypertensive medications). Twin phenotyping is described below.

Environmental (cold) stress in twin pairs. A total of 149 twin pairs were studied; each subject was self-identified as being of white (European) ancestry. BP was recorded continuously and noninvasively in seated subjects with a radial artery applanation tonometer and dedicated sensor hardware (Colin Pilot, Colin Instruments) and software (ATLAS, WR Medical, Stillwater, Minnesota; TDA [Tonometric Data Analysis], Colin Instruments). During the cold pressor test, after a 10-min equilibration period, the left hand was immersed in ice water (at 0°C) for 60 s, as previously described (28,31). The device was periodically calibrated against an automated cuff BP in the contralateral arm. Heart rate was similarly recorded with thoracic electrocardiogram electrodes. We identified at least 3 beats with consistent (within 10%) values for BP and heart rate just before and at the end of the cold pressor test, and resulting changes in BP and heart rate were recorded. Even though BP may continue to rise at 2 to 3 min of cold exposure (32), we chose the 1-min time point since in longitudinal studies this 1-min BP increment has been shown to predict the development of hypertension decades later (33).

Biochemical assays. Ethylenediamine tetraacetic acid–anticoagulated plasma was frozen and stored at −70°C before assay. The region-specific radioimmunoassay for $CHGA_{116\text{-}439}$ (precursor) was based on a polyclonal rabbit antiserum (34). ^{125}I-radiolabeling of the protein was enabled by endogenous Tyr residues, as described in detail elsewhere (35,36). Catecholamines were measured by radioenzymatic assay, as previously described (37).

Genomics. Genomic deoxyribonucleic acid (DNA) was prepared from leukocytes in ethylenediamine tetraacetic acid–anticoagulated blood, using PureGene extraction columns (Gentra Biosystems, Minneapolis, Minnesota) as previously described (38).

The reference sequence (RefSeq) for human *CHGA* was obtained from the UCSC Genome Browser. We resequenced the *CHGA* locus (8 exons, intron/exon borders, UTRs, and proximal promoter) for exhaustive variant discovery in 180 subjects (2n = 360 chromosomes), as previously described (21). These subjects included 51 white (European ancestry) subjects.

Single nucleotide polymorphism (SNP) diploid genotypes were scored by either of 2 base-extension systems: the MALDI-TOF system (Sequenom Inc., La Jolla, California) (39) or the luminescent system of Pyrosequencing (Biotage, Uppsala, Sweden) (40). In each case, initial polymerase chain reaction (PCR) amplification of the template was followed by primer-mediated base extension across the variant position. Pyrosequencing primers were designed using the dedicated software provided by Pyrosequencing (Uppsala, Sweden). Target sequences were amplified by PCR from 15 ng genomic DNA in a final volume of 10 μl. To ensure accurate assignment, genotypes were verified by visual inspection, and artifactual data were excluded from further statistical analysis.

Chromaffin cell culture. PC12 rat pheochromocytoma cells (41) were obtained from David Schubert, Salk Institute, La Jolla, California. They were cultured in high-glucose Dulbecco's modification of Eagle's medium with 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum, and penicillin/streptomycin. Cell passage number (since initiation of the line) was between 10 and 25 in these experiments.

Function of *CHGA* 3′-UTR variants: 3′-UTR/luciferase reporter activity assays. The full-length human *CHGA* cDNA previously subcloned into pET21a(+)-hCHGA-His was used as a template. The entire 407 bp of human *CHGA* 3′-UTR was PCR-amplified from this plasmid, and then ligated into the XbaI site of the pGL3-Promoter vector (Promega, Madison, Wisconsin), just downstream (3′) of the luciferase open reading frame. SNPs in the 3′-UTR were recreated by site-directed mutagenesis (QuikChange, Stratagene, La Jolla, California) to reproduce the 3 different naturally occurring variants in the *CHGA* 3′-UTR: C+87T (C11825T), C+96T (C11834T), and C+274T (C12012T). Inserts were sequence-verified (for both orientation and the correct point mutation) before use. Plasmids were purified on columns (Qiagen, Valencia, California) before transfection of supercoiled DNA. PC12 rat pheochromocytoma cells were transfected (at 50% to 60% confluence, 1 day after 1:4 splitting) with 1 μg of each construct mentioned in the preceding text, as well as 10 ng of the Renilla luciferase expression plasmid pRL-TK (Promega), as an internal transfection efficiency control in each well, by the cationic liposome method (Superfect, Qiagen). The firefly and renilla luciferase activities in the cell lysates were measured 24 h after transfection, and the results were expressed as the ratio of firefly/renilla luciferase activity ("Stop & Glow," Promega). Each experiment was repeated a minimum of 3 times.

Role of *CHGA* in catecholamine storage vesicle formation. SMALL INTERFERING RIBONUCLEIC ACID (SIRNA) AND TRANSFECTION. Rat *CHGA*-siRNA oligonucleotides (sense CAACAACAACACAGCACUdTdT, and anti-

sense AGCUGCUGUGUUGUUGUUGdTdT) were synthesized by Dharmacon (Lafayette, Colorado) according to the AA (N19) TT pattern (siRNA selection software, Whitehead Institute, Cambridge, Massachusetts). Annealed siRNA duplexes were resuspended in RNAse-free solution buffered to pH 7.4. PC12 cells were transfected with the indicated amount of siRNA-*CHGA* duplex using RNAiFect transfection reagent (Qiagen) according to the manufacturer's instructions. Silencing of *CHGA* expression was evaluated by immunoblotting, and the effect of reduced expression of *CHGA* on dense-core secretory granule cellular content was examined by transmission electron microscopy.

IMMUNOBLOTTING ANALYSIS. Whole cell lysates were prepared, and expression of *CHGA* and actin was evaluated by SDS-PAGE followed by immunoblotting, using a rabbit polyclonal anticatestatin (rat $CHGA_{352\text{-}372}$) or a monoclonal antiactin (antiactin I-19, Santa Cruz Biotechnology, Santa Cruz, California) primary antibody, followed by horseradish peroxidase conjugate secondary antibodies. Immunoreactivity was visualized by chemiluminescence (Pierce Chemical Company, Rockford, Illinios), and protein expression was quantified by densitometry (NIH image 1.6).

ELECTRON MICROSCOPY. Cells were incubated in modified Karnovsky's fixative (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 mol/l Na cacodylate buffer, pH 7.4) overnight at 4°C, followed by 1% OsO_4 in 0.1 mol/l Na cacodylate buffer, pH 7.4 and subsequently dehydrated using a graded series of ethanol solutions followed by propylene oxide and infiltration with epoxy resin. After polymerization at 65°C overnight, thin sections were cut and stained with uranyl acetate (4% uranyl acetate in 50% ethanol) followed by bismuth subnitrate. Sections were examined at an accelerating voltage of 60 kV using a Zeiss EM10B electron microscope.

Statistical analyses. Results are presented as the mean value ± SEM.

HAPLOTYPES AND POPULATION BP EXTREMES. Pairwise linkage disequilibrium (LD) between each common SNP pair across the *CHGA* locus was quantified as D′ by the GOLD (Graphical Observation of Linkage Disequilibrium) software (42). Comparison of haplotype frequencies between population BP extremes (hypertensive cases vs. control subjects) was performed using the single nucleotide polymorphism expectation maximation (SNPEM) algorithm (43). SNPEM estimates haplotype frequencies for each group using the EM algorithm, taking into account the probability of all possible haplotype pairs, and calculates a likelihood statistic to compare haplotype frequencies between 2 groups (cases vs. control subjects) and a permutation test to determine significance in the face of multiple comparisons (set at 10,000 permutations). SNPEM was used to perform a "sliding window" analysis to identify associated haplotype lengths (from 1 to 4 SNPs) across the locus (44), thus evaluating all possible haplotypes across the 4 SNPs, thereby interrogating genetic variation at the locus in an unbiased, hypothesis-free way. Potential shortcomings of this method include limitation of analysis to dichotomous traits; a focus on the chromosome, rather than the individual, as the unit of analysis; no measure of direction or magnitude of a genetic effect (other than p value); and lack of adjustability for potential confounding covariates. The second approach utilized haplotype assignment to individuals. HAP (version 3.0) was used to impute haplotypes from diplotype genotypes (45). This 2-step analysis approach was implemented to avoid limitations of the first method and potential inflation of type I (false positive) errors introduced by haplotype inference and assignment in the second method (44). The 2 highest probability haplotypes were assigned to each individual and used in marker-on-trait analyses; haplotype assignment was not further modeled for probability or uncertainty. Haplotypes were then used as independent variables in general linear model tests, such as 1- or 2-way analysis of variance (ANOVA). Additional permutation tests (46) on 3 × 2 contingency tables (diploid genotype vs. BP status) were used to confirm genotype effect on the dichotomous BP trait.

To account for multiple comparisons resulting from 4 SNP loci typed across the *CHGA* locus in the population BP extremes, 3 methods were used. First, a conservative Bonferroni correction was employed, assuming independence of genotypes across the 4 positions, yielding a modified alpha threshold of 0.05/4 = 0.0125. Second, the method of SNP spectral decompositon was used (47), to account for correlations among the SNPs. The third method was use of the principles of false discovery rate, using the Simes procedure (48) as outlined by Benjamini and Hochberg (49), which assumes independence of the 4 SNP genotypes, yielding a modified alpha threshold of alpha = (m + 1)/2m, where m = the number of independent tests.

TWIN PAIRS. Estimates of heritability (h^2) in twin pairs were obtained using the variance-component methodology implemented in the SOLAR (Sequential Oligogenic Linkage Analysis Routines) package (50). Some of the h^2 values (e.g., cold stress) in twin pairs have been reported previously (28). Descriptive statistics (means ± SEM) were computed for genotype groups across both members of each twin pair, using generalized estimating equations, in SAS (Statistical Analysis System, Cary, North Carolina), establishing an exchangeable correlation matrix to take into account intratwin-pair correlations (51). Data were stored in Microsoft Access, and analyses were conducted in SPSS (Statistical Package for the Social Sciences, Chicago, Illinois), SAS, or SOLAR. If traits were not normally distributed, values were $\log_{10}$-transformed to decrease skewness, or tested by nonparametric methods (Kruskal-Wallis or median tests).

3′-UTR motifs. Messenger ribonucleic acid (RNA) (3′-UTR) motifs differing between SNP variants were examined at

Table 1 *CHGA* Haplotypes Across the Locus in Subjects With Extreme BP Values in the Population*

Variant	Position to Cap Site (+/−)	Domain	Major Allele (%)	Minor Allele (%)	Hardy-Weinberg Equilibrium Chi-Square	p Value
G-462A	−462	Promoter	G (76.7)	A (23.3)	9.20	0.002
Glu246Asp	+8,540	Coding, exon-6	Glu (92.0)	Asp (8.0)	0.76	0.384
Arg381Trp	+9,610	Coding, exon-7	Arg (86.3)	Trp (13.7)	1.39	0.238
C+87T	+11,825	3′-UTR, exon-8	C (73.5)	T (26.5)	0.260	0.610

*Characteristics of the individual variants. Base positions are numbered (+/−) with respect to the cap site (exon-1, or transcriptional start site).
Arg = arginine; Asp = aspartic acid; BP = blood pressure; *CHGA* = chromogranin A; Glu = glutamic acid; Trp = tryptophan; 3′-UTR = 3′-untranslated region.

RegRNA, an integrated web server for identifying regulatory RNA motifs and elements, including miRNA motifs (52). Likely mRNA stabilities (from energy-minimized 3′-UTRs) between 3′-UTR variants were estimated with the algorithm RNAfold (53). One-way ANOVA with least significant difference post hoc correction was performed on in vitro SNP-specific 3′-UTR activities.

Results

Genetic case/control study in BP extremes from the population. Here we studied whether allelic or haplotypic variation at *CHGA* predicts BP elevation.

SLIDING WINDOW HAPLOTYPE ANALYSIS: THE DICHOTOMOUS BP TRAIT (HYPERTENSION). After systematic variant discovery at *CHGA*, we first evaluated 20 common SNPs (each with minor allele frequency >10%) distributed across ~13 kbp at the locus, to probe patterns of pairwise LD in subjects of European (2n = 102 chromosomes) ancestry. (Online Table 1). Within this population, the 20 common SNPs were generally tightly linked, with D′ >0.9 across the entire locus (Online Fig. 1).

Since pairwise LD analysis demonstrated high LD across the *CHGA* locus in the white population, we used SNPEM to impute the most likely haplotypes across the entire locus. We selected 4 common (minor allele frequency 8% to 26%) SNPs in several functional domains (promoter, coding, and UTR) to span the locus (Table 1): promoter G-462A, coding/exon-6 Glu246Asp, coding/exon-7 Arg381Trp, and 3′-UTR/exon-8 C+87T (C11825T). One (single)-SNP, 2-SNP, 3-SNP, or 4-SNP haplotype associations were then tested for association with hypertension, in the population of BP extreme individuals (Online Table 1). We report p values from omnibus permutation tests in SNPEM (54). *CHGA* SNPs and haplotypes displayed sex-dependent effects on BP status (Fig. 1A). In the entire group (men and women), significant associations were found for the 4-SNP haplotype ($p = 0.003$) as well as the 3′-UTR alone ($p = 0.043$). Associations of *CHGA* SNPs and haplotypes were substantially more prominent in men than women. In men, 1-, 2-, 3-, and 4-SNP haplotypes associated with BP (all $p < 0.05$), with the most prominent associations clustering toward the 3′-end of the gene. For single SNPs, the peak association was at the 3′-UTR (C+87T, $p = 0.005$).

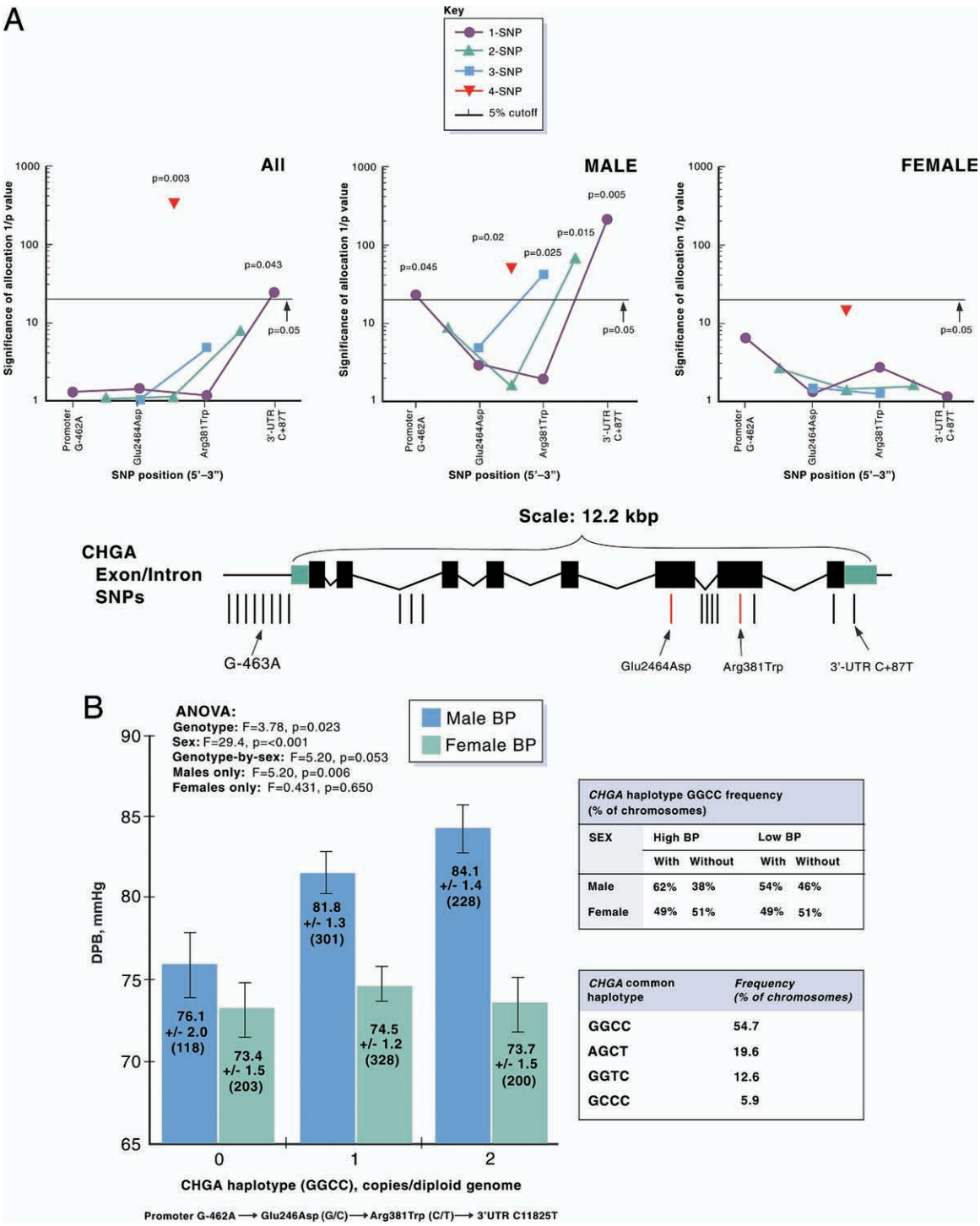
An overly conservative Bonferroni correction for 4-SNP loci yields a target alpha of $(0.05/5) = 0.0125$, while a false discovery rate Simes-adjusted alpha for 4 tests would be $0.05(4 + 1)/2 \times 4 = 0.03125$, and by SNP spectral decompositon (47), the experiment-wide significance threshold required to keep type I error rate at 5% is 0.0134. Each of these thresholds is exceeded in men. In women, none of the haplotypes (or single SNPs) offered significant prediction of BP status (all $p > 0.05$).

Characteristics of the 4 SNP genotypes are presented in Table 1. Although promoter G-462A deviated from Hardy-Weinberg equilibrium, all 3 genotype classes were represented in the data (G/G = 715 [60.4%]; G/A = 386 [32.6%]; A/A = 83 [7.0%]), and visual inspection of the Pyrosequencing base-extension luminescence tracings revealed robust/unequivocal evidence of incorporation of each base (G vs. A, on the antisense [−] strand).

***CHGA* HAPLOTYPES AND THE QUANTITATIVE BP TRAIT.** Haplotype GGCC (promoter −462G→246Glu/G→381Arg/C→3′-UTR/+87C), the most common haplotype at the locus (Fig. 1B), dose-dependently predicted higher DBP in men ($p = 0.006$), though not women ($p = 0.650$). Thus, in the context of haplotype GGCC, the C (major) allele at 3′-UTR C+87T progressively elevated DBP in men.

***CHGA* 3′-UTR C+87T AND THE DICHOTOMOUS AS WELL AS CONTINUOUS BP TRAIT (HYPERTENSION).** During sliding-window analysis, the 3′-UTR variant displayed the most prominent effect on BP status. In men considered alone, hypertensive and normotensive individuals differed significantly in C+87T (C11825T) diploid genotype frequencies ($p = 0.005$), though a genotype effect was not seen in women alone ($p = 0.158$) (Table 2, top, Online Fig. 2A). Permutation tests (on 3 × 2 contingency tables, diploid genotype vs. BP status) confirmed the genotype effect in men ($p = 0.015$), and lack of effect in women ($p = 0.363$).

In Table 2, bottom (Online Fig. 2B), we show the effects of C+87T on SBP and DBP as continuous traits (in mm Hg). Even though these subjects were ascertained from the population on a DBP criterion, the hypertensive individuals exhibited elevations of both DBP and SBP (Online Table 2), and there were significant effects of C+87T genotype on both SBP ($p = 0.028$) and DBP ($p = 0.037$). Likewise, there were significant genotype-by-sex interactions on both SBP ($p = 0.015$) and DBP ($p = 0.010$). In men, BP differed substantially between homozygote (C/C, T/T) classes: by



CHGA haplotype GGCC frequency (% of chromosomes)

SEX	High BP		Low BP	
	With	Without	With	Without
Male	62%	38%	54%	46%
Female	49%	51%	49%	51%

CHGA common haplotype	*Frequency (% of chromosomes)*
GGCC	54.7
AGCT	19.6
GGTC	12.6
GCCC	5.9

Figure 1 ***CHGA* Common Haplotypes and BP in the Population**

(A) Chromogranin A *(CHGA)* polymorphisms in individuals from population blood pressure (BP) extremes: haplotype sliding-window analysis. Four common variants (each minor allele frequency ≥8%) (Table 1) were scored to span the *CHGA* locus. The exon/intron structure of the locus, as well as the positions for all common single nucleotide polymorphisms (SNPs) (21), is depicted in the schematic at the **bottom**. Results for haplotypes composed of 1, 2, 3, or 4 SNPs were computed by the SNP expectation maximization algorithm for the dichotomous BP trait, and significance is plotted as reciprocal p values for each group: all subjects **(left)**, men only **(center)**, and women only **(right)**. The p values were derived from omnibus permutation tests. Significant (<0.05) p values are shown at the appropriate point. **(B)** *CHGA* common extended haplotype GGCC: sex-dependent effect on BP as a quantitative trait in population BP extremes. Extended 4-SNP haplotype GGCC is the most common haplotype in this population (at 57.4% of chromosomes; see inset). The effect of haplotype GGCC on the quantitative trait diastolic blood pressure (DBP) is illustrated separately for men and women. There is a significant overall effect for genotype ($p = 0.023$), as well as an effect in men alone ($p = 0.006$).

Table 2 *CHGA* 3′-UTR Common Variant C+87T: Effect of the T Allele on BP Status and BP Values*

Sex	Genotype	BP-High (n)	BP-Low (n)	p Value (2-Sided)
CHGA C+87T genotype frequencies and BP status (dichotomized)†				
Male	C/C	156	107	
	C/T or T/T	95	112	0.004
Female	C/C	112	170	
	C/T or T/T	119	148	0.262

Sex	Genotype	SBP (mm Hg)	DBP (mm Hg)
CHGA C+87T and BP as a continuous trait‡			
Male	C/C	137.2 ± 1.6	83.7 ± 1.3
	C/T or T/T	131.2 ± 1.8	77.6 ± 1.5
	ANOVA p value	0.016	0.003
Female	C/C	123.8 ± 1.7	71.8 ± 1.2
	C/T or T/T	126.9 ± 1.7	73.8 ± 1.3
	ANOVA p value	0.206	0.272

*Subjects are grouped by absence (C/C) or presence (T/T or C/T) of the T (minor) allele; †results evaluated by chi-square test; ‡allelic association (presence or absence of the T allele) evaluated by univariate analysis of variance (ANOVA).
DBP = diastolic blood pressure; SBP = systolic blood pressure; other abbreviations as in Table 1.

~12 mm Hg for SBP and ~9 mm Hg for DBP. By ANOVA (adjusted) R^2 in men, C+87T accounted for ~1.9% of the population variance in SBP (or ~13.7 mm Hg), and ~1.2% of the variance for DBP (or ~5.8 mm Hg). In women, C+87T did not affect either SBP or DBP.

To focus on the role of the minor (T) allele, we combined the minor allele homozygotes (T/T) with heterozygote (C/T) categories, and compared them with C/C homozygotes. The C allele retained its significant effect on BP in men ($p = 0.003$ to 0.016), though not in women.

Because women in the BP extreme samples spanned a range of ages, we then divided the women about the approximate age of human menopause (±50 years); there was no effect of C+87T on BP in either the older (age ≥50 years; $p = 0.067$ for SBP and $p = 0.066$ for DBP) or younger (age <50 years; $p = 0.187$ for SBP and $p = 0.379$ for DBP) women, nor was there an age-by-sex interaction ($p = 0.879$ for SBP and $p = 0.815$ for DBP).

Human *CHGA* expression in vivo. HERITABILITY (H^2) IN TWIN PAIRS. We used a phenotyped twin-pair cohort to estimate the influence of heredity on *CHGA* secretion (Table 3). Plasma *CHGA* concentration showed significant ($p < 0.0001$) heritability in twins, at 45 ± 12% for the precursor (epitope: $CHGA_{116\text{-}439}$).

***CHGA* AND NOREPINEPHRINE SECRETION: EFFECTS OF HYPERTENSION AND SEX.** We tested whether *CHGA* gene expression varied in patients with essential hypertension, evaluating the plasma *CHGA* precursor (epitope: human $CHGA_{116\text{-}439}$). Plasma *CHGA* was substantially higher in hypertension ($p < 0.001$) (Fig. 2A, Online Table 2), but a significant ($p < 0.001$) BP status-by-sex interaction largely confined the increase to male hypertensives. The results suggest increased *CHGA* biosynthesis and exocytotic secretion especially in men with hypertension. Plasma norepinephrine was also elevated in hypertension ($p < 0.001$) (Fig. 2B, Online Table 2), but once again a significant BP status-by-gene interaction ($p = 0.002$) restricted the effect mainly to men.

***CHGA*: PREDICTOR OF CATECHOLAMINE SECRETION.** When we subdivided individuals into 2 (upper and lower) *CHGA* quantiles by dividing about the median value (Fig. 2C), individuals with greater *CHGA* secretion also exhibited increased plasma norepinephrine ($p = 0.0399$) and epinephrine ($p = 0.0007$) secretion.

***CHGA* 3′-UTR C+87T (C11825T) CIS-QTL AND PLASMA *CHGA*.** Since *CHGA* C+87 affected BP, and the *CHGA* plasma level is substantially higher in hypertensive than normotensive individuals (Fig. 2A), the relationship between C+87T genotype and *CHGA* plasma level was explored. Figure 2D shows that common variant *CHGA* C+87T predicts human plasma $CHGA_{116\text{-}439}$ concentrations; increasing numbers of the T allele (0→1→2) progressively reduced *CHGA* by ~10% ($p = 0.007$). No gene-by-sex effect was found ($p = 0.57$).

***CHGA* genotype and environmental (cold) stress: studies in twin pairs.** Since systemic hypertension may result from the cumulative effects of transient adverse BP responses to environmental stress in genetically predisposed individuals (55), we probed the BP response to systematic cold stress (28) in a series of predominantly normotensive twin pairs. The stress BP trait is significantly heritable ($h^2 = 29 \pm 8\%$; $p < 0.0001$), as estimated by twin-pair variance components (28) (Table 3). The common *CHGA* 3′-UTR variant C+87T (C11825T) predicted final (post-cold) SBP ($p = 0.042$) during this environmental stressor (Fig. 3A): increasing numbers of the minor (T) allele seemed to blunt the final SBP response, while the basal SBP pre-cold stress was almost the same between the 3 genotype groups (C/C group: 119.4 ± 1.6 mm Hg; C/T group: 119.8 ± 1.6 mm Hg; and T/T group: 114.3 ± 2.7 mm Hg; $p = 0.21$). Sex also influenced post-stress SBP: men had ~6 mm Hg higher values for post-stress SBP than women ($p = 0.009$) (Fig. 3B), though there was no gene-by-sex interaction on this BP trait ($p = 0.185$). While initial (pre-cold) SBP also predicted final (post-cold) SBP (Spearman $p = 0.713$, $p <$

Table 3 Heritability of "Intermediate" Traits in Twin Pairs*

Trait	h^2 as % (± SEM)	p Value
Biochemical		
Plasma $CHGA_{116\text{-}439}$ (precursor)	45 ± 12	<0.0001
Physiological		
Cold stress final SBP	29 ± 8	<0.0001

*Heritability ($h^2 = V_G/V_P$, or the fraction of phenotypic variance accounted for by genetic variance) was determined by variance components analysis in the Sequential Oligogenic Linkage Analysis Routines.
CHGA = chromogranin A; SBP = systolic blood pressure.

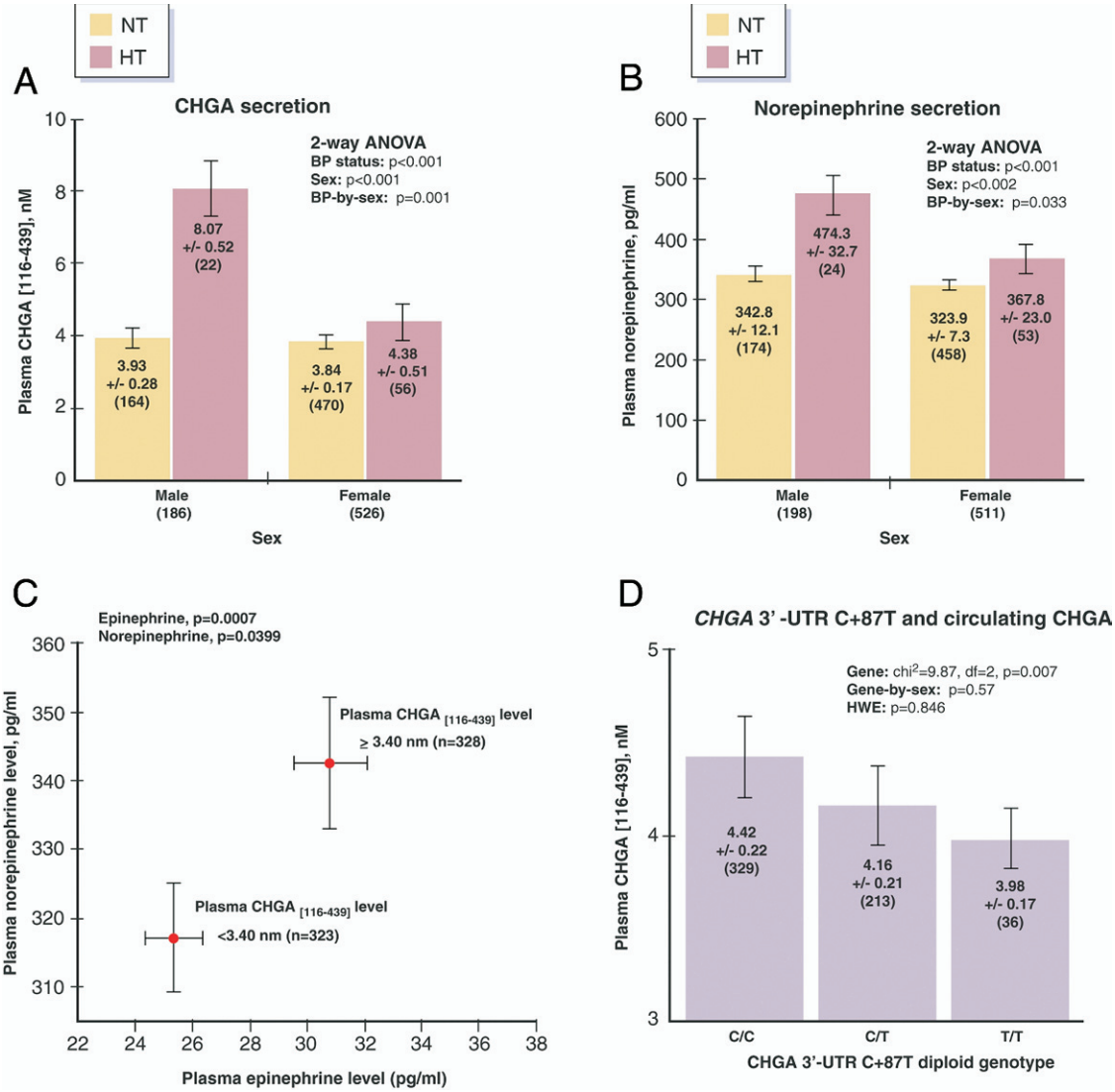


Figure 2 ***CHGA* and Catecholamine Secretion: BP, Sex, and the 3′-UTR**

(A) Hypertension, sex, and *CHGA* secretion. Resting plasma concentration of the *CHGA* precursor (epitope: $CHGA_{116\text{-}439}$) was measured in plasma from seated human subjects with normal renal function (serum creatinine ≤1.5 mg/dl). We studied subjects with a diagnosis of essential hypertension (HT), versus unmedicated control subjects (NT) with normal blood pressure (BP). **(B)** Hypertension, sex, and catecholamine secretion. The same individuals were studied for plasma norepinephrine concentration (*CHGA* and norepinephrine were measured in the same plasma sample). **(C)** *CHGA* as a predictor of catecholamine secretion. Individuals were divided into quantiles above and below the median value for plasma *CHGA* concentration, and plasma catecholamine concentrations were calculated in the 2 groups (*CHGA* and catecholamine were measured in the same plasma sample). **(D)** *CHGA* 3′-untranslated region (3′-UTR) variant C+87T: influence on circulating *CHGA*. Resting plasma concentration of the *CHGA* precursor (epitope: $CHGA_{116\text{-}439}$) was measured in plasma from 578 genotyped white subjects (187 men, 391 women). Each subject had normal renal function (serum creatinine ≤1.5 mg/dl). Since the distribution of plasma $CHGA_{116\text{-}439}$ concentration in this sample deviated substantially from normality (skewness = 6.03 ± 0.10, kurtosis = 55.2 ± 0.20), we used a nonparametric median test (evaluating whether 2 or more independent samples [defined by diploid genotype] are drawn from populations with the same median, using the chi-square statistic). There was no gene-by-sex interaction on the *CHGA* trait (p = 0.57). HWE = Hardy-Weinberg equilibrium; other abbreviations as in Figure 1.

0.001), it did not predict the cold-induced change in SBP (Δ = [final-initial]; Spearman p = 0.074, p = 0.156).

Function of *CHGA* 3′-UTR variants. SEQUENCE CONSERVATION AND PHYLOGENY AT *CHGA* 3′-UTR COMMON VARIANT C+87T. Figure 4A depicts the *CHGA* 3′-UTR sequence across species in the region of 3 naturally occurring variants (21): common variant C+87T (C11825T, global minor allele frequency across populations, 17.7%), as well as the less common variants C+96T (C11834T, minor allele frequency = 0.6%) and C+274T (C12012T, minor allele frequency = 0.6%). The local genomic region at and around C+87T is highly conserved across species; at 3′-UTR position +87, C or T are the only alleles found in mammals. Similar local sequence conservation across mammals is found around the positions of the more unusual 3′-UTR variants C+96T and C+274T. Based on the chimpanzee

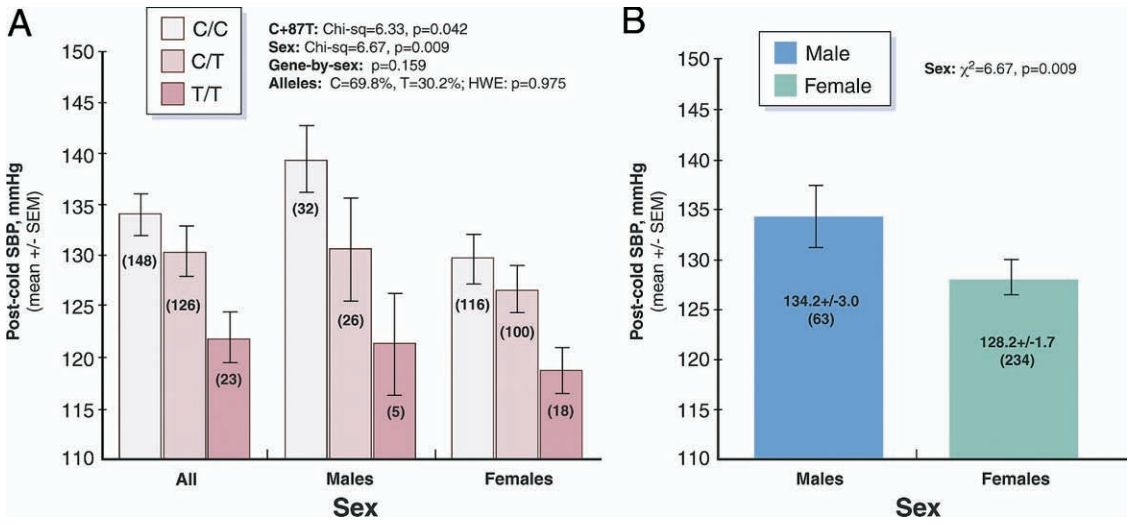


Figure 3 **BP Response to Environmental (Cold) Stress in Twin Pairs: Effect of *CHGA* Common Allelic Variant C+87T in the 3′-UTR, as Well as Sex**

Provocation of efferent sympathetic outflow was undertaken in each subject by immersion of 1 hand in ice water (at 0°C) for 1 min, with continuous blood pressure (BP) monitoring. Results are shown for final (post-cold) systolic blood pressure (SBP), and analyzed by generalized estimating equations, establishing an exchangeable correlation matrix to take into account intratwin-pair correlations. **(A)** *CHGA* C+87T genotype effect on post-stress SBP; data are shown in all persons, as well as men and women separately. Genotype affected the trait ($p = 0.042$), and there was no gene-by-sex interaction ($p = 0.159$) on trait. **(B)** Sex effect on post-stress SBP ($p = 0.009$). 3′-UTR = 3′-untranslated region.

sequence, C+87 is likely to be the ancestral +87 allele in the human population (56), though another primate (*Macaca*) also displays the +87T allele.

IN CELLA REPORTER GENE ACTIVITY ASSAY. To directly investigate the role of natural genetic variation of the *CHGA* 3′-UTR in *CHGA* expression, we constructed plasmids in which we monitor the influence of the 3′-UTR on a luciferase reporter. The full 407 kbp human *CHGA* 3′-UTR, containing 1 of 4 variant (wild-type vs. 1 common or 2 uncommon SNPs) sites, was inserted downstream (3′) of the luciferase gene in the pGL3 promoter vector. After transfection into PC12 chromaffin cells, reporter gene activity was measured at 24 h. The wild-type 3′-UTR haplotype (C+87→C+96→C+274) increased reporter gene activity (compared with the vector without the 3′-UTR insert), while the T allele at any of these 3 positions decreased reporter gene activity (as compared with the wild-type haplotype); the common +87T variant alone decreased expression by ~30% ($p = 0.009$) (Fig. 4B). These in vitro results document that common SNP C+87T is a functional polymorphism, and the in vitro effect of the +87T allele to diminish expression is consistent with the in vivo observations that +87T is associated with diminished plasma *CHGA*, stress BP response, and basal BP in the population.

***CHGA* 3′-UTR VARIANTS: COMPUTATIONAL BIOLOGY.** C+87T is not located within a known motif that influences mRNA stability, such as an A/U (A/T)-rich region (Fig. 4A), and does not create or abolish other motifs such as micro-RNA recognition sites (52), nor were there changes in folding energy ($\Delta G^{o} = -147.58$ kcal/mol for C+87 vs. −147.42 kcal/mol for +87T), and the 2 mRNAs did not differ in overall stem/loop structure (53).

Disease mechanisms: role of *CHGA* in catecholamine storage vesicle formation. To probe how quantitative alterations in *CHGA* gene expression might affect sympathoadrenal structure or function, we "silenced" *CHGA* expression in chromaffin (rat PC12) cells using the specific technique of siRNA targeting the rat *CHGA* mRNA. The *CHGA* siRNA reagent dose-dependently suppressed *CHGA* protein expression by up to ~95%, without affecting expression of the control (structural or "housekeeping") protein actin (Figs. 5A and 5B). In control cells (treated with "mock" siRNA reagent), ultrastructural examination revealed numerous chromaffin granules, largely "docked" at the cytoplasmic surface of the plasma membrane (Fig. 5C). Application of the *CHGA* siRNA resulted in the disappearance of the majority of the chromaffin granules from the cell (Fig. 5D); on a quantitative basis, the number of dense-core granules per cell X-Y cell plane declined by approximately two-thirds ($p < 0.0001$) (Fig. 5E). Ultrastructural morphology of the chromaffin cells (nuclei, mitochondria, endoplasmic reticulum) was not otherwise disturbed, reinforcing the specificity of the change in chromaffin granules.

Discussion

Overview. *CHGA* plays a pivotal role in the sympathochromaffin system, both in the formation of catecholamine

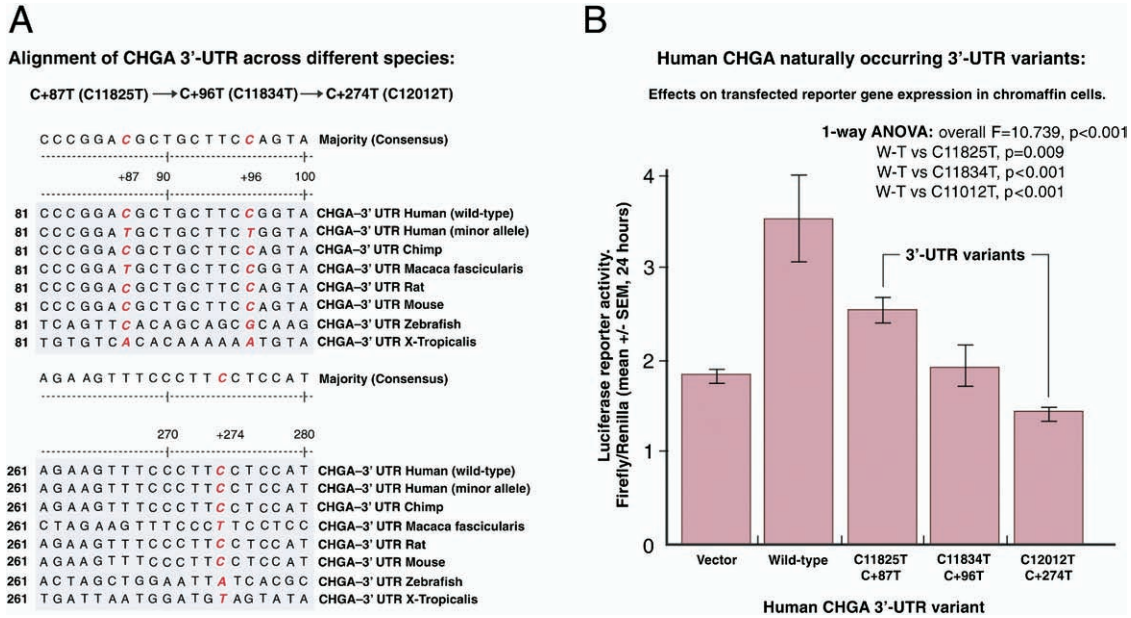


Figure 4 **Function of *CHGA* 3′-UTR Variants**

(A) Sequence alignment of the *CHGA* 3′-untranslated region (3′-UTR) across different species. 3′-UTR sequences are aligned across 7 species, to illustrate conservation at positions of 3 naturally occurring human polymorphisms: C+87T (C11825T), C+96T (C11834T), and C+274T (C12012T). **(B)** Human *CHGA* naturally occurring 3′-UTR variants: effects on transfected reporter gene expression in chromaffin cells. The 3′-UTR variants tested were: C+87T (C11825T), C+96T (C11834T), and C+274T (C12012T). Results of luciferase activity measurements 24 h after transfection of the 4 versions of the 407-bp *CHGA* 3′-UTR (wild type [W-T] and 3 variants) into chromaffin cells. Results are expressed as the ratio of firefly luciferase/Renilla luciferase (encoded by the transfection efficiency plasmid pRL-TK). Each experiment was performed in triplicate, and such experiments were repeated at least twice. The W-T contains the major (C) allele at all 3 positions. Variant constructs contain the minor (T) allele at the indicated position, but the major (C) at the 2 other positions.

secretory vesicles and in the regulation of transmitter release (1,20). In this report we approach the impact of common human variation at the *CHGA* locus for autonomic physiology and disease. We found that *CHGA* is overexpressed in hypertension, and that a common (~27% frequency) genetic variant in the *CHGA* 3′-UTR (C+87T) is strongly associated with human essential hypertension, accounting for up to ~12/~9 mm Hg of BP variation within the population. The 3′-UTR variant also predicts environmental stress-induced increments in BP, suggesting a mechanism for early effects of the gene on a pathogenic series of events eventuating in sustained BP elevation. The 3′-UTR variant is in a region of sequence conservation across species, and acts to change *CHGA* gene expression in chromaffin cells, perhaps eventuating in diminution of catecholamine secretory granules; thus, *CHGA* C+87T fulfills many criteria for a functional variant contributing to disease predisposition. At multiple levels (*CHGA* expression, heritable circulatory response to environmental stress, and finally basal BP in the population), sex seemed to play an important role in mediating the effect of genetic variation on phenotype.

Sex: role in hypertension and intermediate phenotypes. In the wake of the sex-dependent effect of *CHGA* genetic variation on BP, we performed a series of studies to explore the interaction of gene and sex, and found that sex played a role at each of several steps. At a *biochemical* level, *CHGA* and norepinephrine secretion were elevated in hypertension, but the increase seemed to be confined to men. At a *physiological* level, the pressor response to environmental stress was influenced by both sex and C+87T genotype. Finally, at a *disease* level, sex was again crucial: in individuals with the most extreme BPs in the population, associations with C+87T were substantially more impressive in men than in women, effectively confining the effect of C+87T to men.

Why might adrenergic genetic variation yield such different consequences in men and women? Acute vascular responses to adrenergic stimuli are sex-dependent (57,58), and the long-term consequences of repeated stressors on resting BP or the late appearance of hypertension differs by sex; for example, in the longitudinal CARDIA (Cardiovascular Reactivity to Video Game Predicts Subsequent Blood Pressure Increases in Young Men) study of cardiovascular risk in young adults (59), reactivity to cold stress predicted 5-year rise in BP and earlier development of hypertension, but only among men and not women. Sex steroids may mediate such differences in BP regulation (33,60–65), though we did not observe an effect of C+87T in either pre- or post-menopausal women.

Haplotypes versus individual variants. Haplotypes are a useful tool for scanning large genomic regions in the search

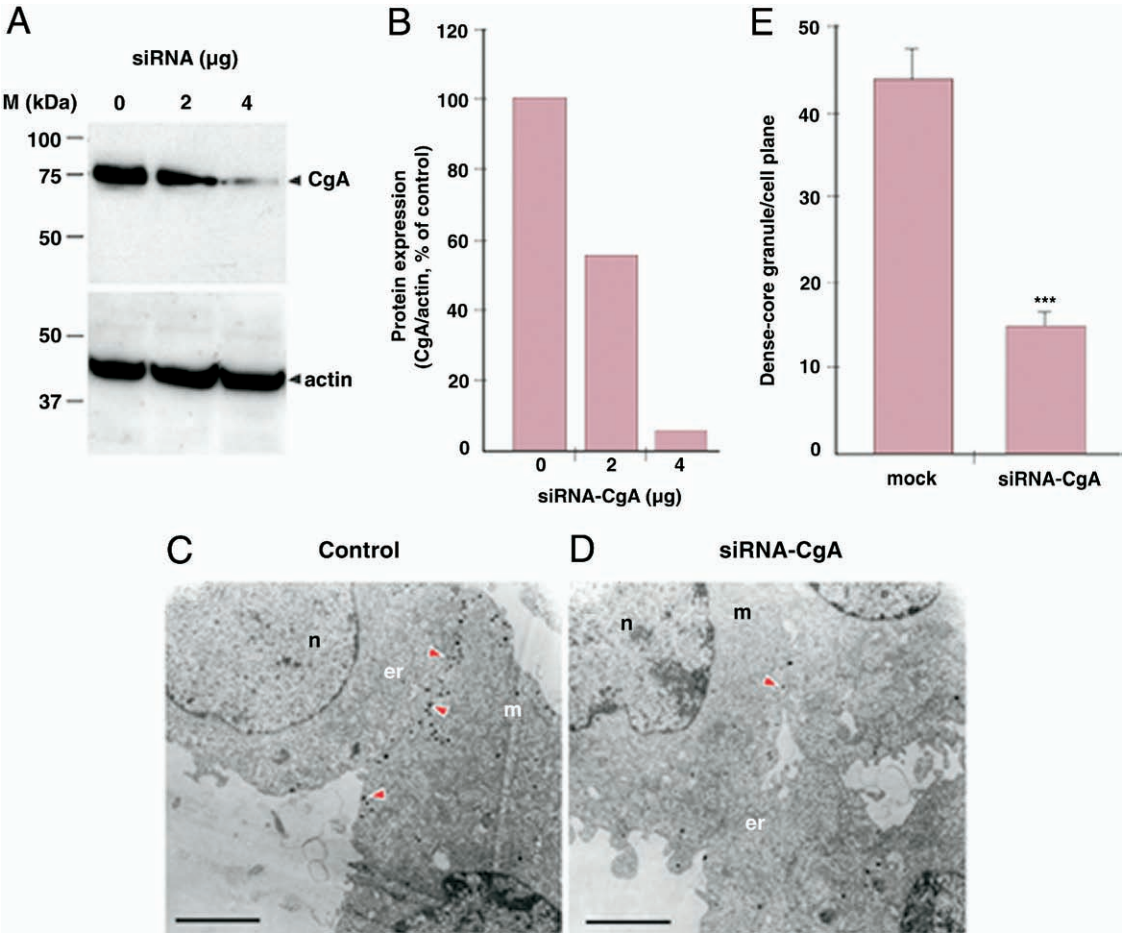


Figure 5 **Disease Mechanisms: Role of *CHGA* in Catecholamine Storage Vesicle Formation**

Effect of small interfering RNA (siRNA) to "silence" expression of *CHGA* protein in sympathoadrenal PC12 cells. PC12 cells were grown after transfection with the indicated amount of 22-bp siRNA-rat *CHGA* duplexes at 4 μg/well for 72 h. **(A)** Visualization of *CHGA* immunoreactivity by chemiluminescent immunoblot. Whole cell lysates were prepared, and expression of *CHGA* and actin (control, "housekeeping" protein) was evaluated by SDS-PAGE followed by immunoblotting using a rabbit polyclonal anticatestatin (rat $CHGA_{367\text{-}387}$) antibody or a monoclonal antiactin primary antibody. **(B)** Densitometry to quantify *CHGA* and actin protein expression, using NIH image v1.6 software. **(C and D)** Electron microscopy. Ultrastructural examination of the effect of siRNA *CHGA* "silencing" on dense-core granule biogenesis in sympathoadrenal PC12 cells. Cells were grown in the presence of 22-bp siRNA-rat *CHGA* duplexes **(D)**, or mock/control **(C)** at 4 μg/well for 72 h. Aldehyde-fixed cells were processed for electron microscopy as described in the Methods section. Dense core granules **(arrowheads)** are seen either "docked" to, or in the vicinity of the plasma membrane. Note that fewer secretory granules are present in the cytoplasm of siRNA-rat *CHGA*-treated cells. **(C)** Control (mock-treated) PC12 cells. **(D)** *CHGA* siRNA-treated PC12 cells. Scale bar: 500 nm. **(E)** Quantification of the abundance of dense-core secretory granules reveals a decreased number of granules per cell planes, as defined by the number of granules found in an XY section of the midcell body. n = 40 for both populations of cells. ***$p < 0.0001$, by *t* test. er = endoplasmic reticulum; m = mitochondria; n = nucleus.

for disease predisposition variants (66). Once a contributory genetic locus has been identified, systematic variant discovery may then yield the underlying polymorphism. At *CHGA*, resequencing (21) identified 1 common variant in the 3′-UTR: C+87T (minor allele frequency, 26.5%). During the initial hypertension association, the peak association for BP in men was far more significant for C+87T ($p = 0.005$) than for haplotypes extending across the entire locus ($p = 0.045$); even haplotype associations tended to peak towards the 3′-end of the locus. When haplotypes were associated with the BP quantitative traits, common haplotype GGCC accounted for ~8 mm Hg of DBP variation in men; by contrast, 3′-UTR variant C+87T accounted for ~11 mm Hg of DBP variation in men. Thus, after systematic polymorphism at a locus, an individual SNP explained a greater proportion of BP variation, and at higher significance, than did haplotypes. Later we established the functional significance of the 3′-UTR SNP in vitro.

Intermediate phenotypes. Essential hypertension is a complex trait (13), with multiple contributory factors derived from both genes and environment. In the setting of late penetrance of the ultimate disease trait (such as hypertension), as well as likely genetic heterogeneity, the "intermediate phenotype" (67) strategy may be a useful approach in the search for disease predisposition loci. Autonomic traits with heritable determination may be of particular

value in investigation of the genetic underpinnings of hypertension. In accordance with this pathway concept, we pursued intermediate traits in this study. Secretion of *CHGA*, estimated by its plasma concentration, is not only elevated in hypertension ($p < 0.001$), but also influenced by C+87T genotype ($p = 0.007$). The hemodynamic response to environmental (cold) stress may be a predictor of the development of later cardiovascular events, such as hypertension (33,62,64,65,68). Such a response, occurring even before the onset of disease, would be a useful physiological "intermediate phenotype" in probing the genetic determinants of hypertension (31,67,69). We report the cold stress response in our twins, indicating that both change in SBP and final (post-stress SBP) are heritable, and may be valuable intermediate phenotypic anchor points for hypertension (28).

In a group of predominantly normotensive twin pairs subjected to autonomic phenotyping, *CHGA* 3′-UTR variant C+87T predicted post-stress SBP ($p = 0.042$). The independent sex effect on this trait ($p = 0.009$) suggests that the intermediate phenotype mechanism for this trait may operate differently in men and women; indeed, cold stress-induced rises in BP seem to be more effective predictors of long-term changes in BP in men than in women (59).

***CHGA* 3′-UTR polymorphism: in cella functional assay.** The 3′-UTRs of genes may contain elements that govern mRNA stability, localization, and translatability, which are crucial steps in the pathway from gene to protein (52,70). We found that 3 SNPs in the *CHGA* 3′-UTR, and C11825T as the only common one, was relatively conservative among different vertebrate species. Our in vitro reporter gene activity assay provides direct evidence of the functionality of 3′-UTR common variant C+87T, as well as 2 other less common 3′-UTR variants, C+96T and C+274T. At C+87T, the T allele diminished expression of the reporter; the T allele was also associated with lower stress BPs in twin pairs, as well as lower basal SBP and DBP in the population.

What is the mechanism by which C+87T influences *CHGA* expression? C+87T does not lie in an A/U (A/T)-rich region of the 3′-UTR, and a computational survey of the 3′-UTR (52) did not reveal evidence of other well-known mRNA stability elements at/near C+87T, such as microRNA recognition motifs. Nor did the C+87T transition influence the likely stem/loop secondary structure of the message, or its folding energy (53). Thus, C+897T may lie in a novel, previously unexplored motif. Future studies of cytoplasmic or nuclear protein binding by the motif may better elucidate the nature of its action.

Catecholamine storage vesicles. siRNA knockdown of *CHGA* expression in chromaffin cells in cella substantially diminished catecholamine storage vesicle formation, perhaps because *CHGA* serves to initiate binding and condensation events within the core of the chromaffin granule (4). This suggests a possible mechanism whereby alterations in *CHGA* expression in vivo might influence autonomic function; a decrement in secretory vesicles would result in diminished releasable transmitter stores in response to acute stimulation, such as cold stress. Indeed, basal *CHGA* secretion predicted the plasma concentrations of norepinephrine and epinephrine in vivo. Since repeated adverse pressor responses (55) may ultimately predispose to fixed elevations in BP (33,62,64,65,68), the cold stress results may provide a mechanistic physiological link between genotype and the ultimate disease phenotype.

The changes in chromaffin granule morphology that we noted are consistent with previous observations on *CHGA* depletion in vitro using *CHGA* antisense RNA (6) or siRNA (71), as well as results in chromaffin cells in vivo after targeted ablation of the *CHGA* locus (20).

Study limitations and advantages. Catecholamine storage vesicles store and release other active peptides besides the chromogranins/secretogranins, including the potent vasoconstrictor neuropeptide Y (72) and the enkephalins (73); the genes encoding these other peptides might also harbor alleles that influence BP, but additional genetic loci were beyond the scope of this study. Although our BP extreme groups were ascertained on a DBP criterion, recent evidence indicates that SBP is at least as important a risk factor for target organ damage; we plan future studies to explore the potential effect of *CHGA* polymorphism in isolated systolic hypertension. The prevalence of hypertension varies substantially across ethnic groups; since these initial studies in hypertension dealt primarily with subjects of European descent, it will be important to extend future observations to different biogeographic ancestry groups. *CHGA* is present in the core of secretory vesicles throughout the neuroendocrine system (1); here we explored the significance of *CHGA* polymorphism only for hypertension, and have not yet considered its influence on other endocrine or neurological disease syndromes. Finally, although we have noted sex differences in *CHGA* polymorphism effects on both basal and stress-inducible BP, we do not have a clear molecular understanding of how such gene-by-sex interactions arise.

Genetic studies of complex traits have been plagued by false-positive conclusions. Here we used several complementary experimental and statistical approaches to guard against this possibility. We studied the effects of *CHGA* variation upon not only a disease trait but also earlier "intermediate" phenotypes, confirming the effects of *CHGA* 3′-UTR variation upon BP traits in 2 independent groups (population BP extremes and twin pairs). Statistically, we employed haplotype, correction for nonindependence of SNPs in LD with each other, and permutation approaches to ensure that the conclusions would be robust. Finally, we established a biological role for the trait-associated genetic variant.

Conclusions and Perspectives

Our data suggest that remarkably common (~27% frequency) functional variation in the 3′-UTR of the catechol-

amine secretory vesicle protein *CHGA* confers a change in heritable environmental stress-induced change in BP, as well as sex-dependent risk for hypertension, both diastolic and systolic. Our observations are consistent with the "intermediate phenotype" (67) framework for complex traits. Common variation at *CHGA* alters gene expression, initially changing autonomic tone as evidenced by changes in the heritable response of BP to environmental stress, eventuating over decades in fixed alterations in basal BP.

At several stages, sex seems to be a critical factor in this cascade of events: expression of *CHGA*, the pressor response to environmental stress, and the effect of the 3′-UTR C+87T on the ultimate population profile of BP.

These observations are consistent with the "common disease/common allele" hypothesis for frequent traits in the population (74), and suggest new molecular strategies for probing the pathophysiology, risk, and rational treatment of hypertension.

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Key Words: hypertension ■ chromaffin ■ catecholamine ■ adrenal ■ sympathetic.

APPENDIX

For a supplementary figure on showing the patterns of linkage disequilibrium at the human *CHGA* locus and *CHGA* 3′-UTR common variant C+87T and BP in population, and supplementary tables on the subject characteristics of *CHGA* genotype and BP study and phenotype (CHGA and catecholamine) and BP study, please see the online version of this article.